Table 1 Mutations of genes associated with telomere maintenance identified in patients with aplastic anemia

References	Gene	Number of mutated and screened patients
Vulliamy et al. [10]	*TERC*	2/17 (12%)
Vulliamy et al. [8]	*TERT*	2/80 (2.5%)
Yamaguchi et al. [9]	*TERC*	2/150 (1.3%)
Yamaguchi et al. [7]	*TERT*	7/200 (3.5%)
Savage et al. [50]	*TERF1*	1/47 (2.1%)
	TERF2	1/47 (2.1%)
Liang et al. [6]	*TERT*	2/96 (2.1%)
Walne et al. [51]	*TINF2*	2/111 (1.8%)
Du et al. [3]	*TERT*	4/199 (2.0%)
Du et al. [4]	*TINF2*	6/109 (5.5%)

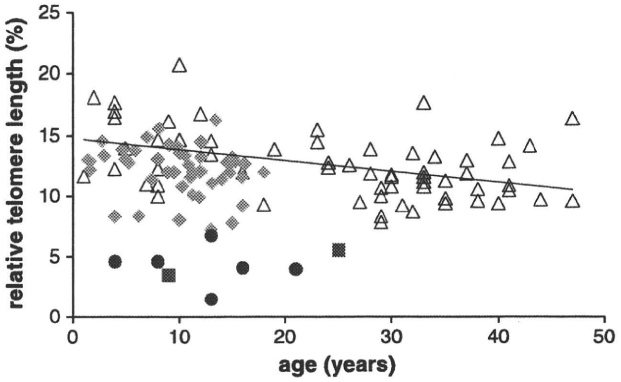

Fig. 2 Relative telomere length in peripheral blood lymphocytes from patients with dyskeratosis congenita (*filled circles*), patients with aplastic anemia harboring *TERT* mutations (*filled squares*), patients with idiopathic aplastic anemia (*filled argyles*) and normal individuals (*open triangles*). Telomere lengths were measured by flow cytometry-fluorescent in situ hybridization (flow-FISH). Relative telomere length was calculated as the ratio between the telomere signal of each sample and the telomere signal of the control cell line (cell line 1301). These data are from the Department of pediatrics, Nagoya University Graduate School of Medicine

hybridization (flow-FISH), flow-FISH is the most appropriate for "prospective" screening [29, 30]. As shown in Fig. 2, patients with DC and AA with the *TERT* mutation demonstrated very short telomeres as compared with idiopathic AA patients and normal individuals. Given the finding that a small subset of patients with apparently idiopathic AA carry telomere gene mutations and recognizing these patients is critical to treatment decisions, it is desirable to screen telomere gene mutations routinely in patients with AA before starting treatment. However, because screening of gene mutations is laborious and time-consuming, we have adopted screening of telomere length in blood cells instead of gene mutations.

It should be noted that short telomeres are not specific for patients with DC but are also seen in patients with bone marrow failure syndromes. Although short telomeres are also found in patients with other congenital bone marrow failure syndromes, such as Shwachman–Diamond syndrome and Fanconi anemia, telomere length in patients with DC is the shortest compared with other bone marrow failure syndromes. In fact, telomere length in most patients with DC is below the first percentile of telomere length found in healthy controls [31].

Family members of patients with DC should receive genetic counseling to rule out if they are silent carriers. In particular, genetic counseling is necessary during the proband search for a donor for hematopoietic stem cell transplantation. Sometimes, telomere length analysis in families with DC demonstrates that mutated carriers with clinical signs of bone marrow failure have the short telomeres. However, telomere length cannot predict the presence or absence of a mutation in family members with bone marrow failure. There are rare cases that show normal telomere length even though they harbor the same mutation as the proband, suggesting that mutation alone does not sufficiently shorten the telomeres [3].

4 Telomere diseases other than bone marrow failure syndromes

Clinical manifestations in patients with DC include not only bone marrow failure, but also other organ failures. Progressive pulmonary fibrosis develops in around 10–15% of patients with DC [17, 18], and is the second most common cause of death. Respiratory failure is also a common fatal complication after hematopoietic stem cell transplantation. Idiopathic pulmonary fibrosis (IPF) is an adult-onset, progressive scarring of the lung of unknown etiology that ultimately leads to respiratory failure. From 2 to 20% of patients with IPF have a family history of the disease that is inherited as an autosomal dominant trait with variable penetrance [12, 32]. Because some individuals in a pedigree of DC had the IPF phenotype, Armanios et al. [12] hypothesized that *TERC* or *TERT* may be candidate genes for familial IPF. They screened 73 probands of IPF and found 6 (8%) had heterozygous mutations in *TERT* or *TERC*. Tsakiri et al. [11] also independently found three missense mutations and one deletion of *TERT* genes in 44 probands of familial IPF and an additional single mutation in 44 sporadic cases of IPF. These mutant telomerase resulted in short telomeres. However, these patients did not show any classic mucocutaneous manifestations of DC.

Liver diseases have been also described as one of the clinical presentations in patients with DC. Some patients with DC develop severe liver complications after hematopoietic stem cell transplantation even if they have a normal liver function at the time of transplant [33]. In parallel with

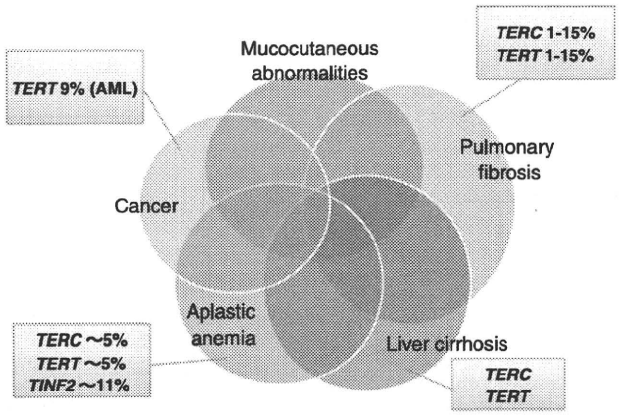

Fig. 3 Schema of phenotypic variations and identified gene mutations in defective telomere maintenance

reports of familial IPF, Calado et al. [13] reported that many relatives of patients with AA and a telomerase mutation had liver diseases, including pathologic fibrosis with inflammation and nodular regenerative hyperplasia. These patients did not present symptoms in childhood or display the characteristic physical abnormalities of DC, but had very short telomeres. These authors proposed that these disorders be collectively considered as "syndromes of telomere shortening". Figure 3 shows the schema of phenotypic variations and identified gene mutations in defective telomere maintenance.

5 Telomere shortening, chromosome instability and cancer predisposition

Patients with DC are prone to hematological malignancies and other solid tumors [17]. The defect of telomere maintenance and telomere attrition leads to chromosomal instability such as loss or gain of chromosomes and end-to-end fusion in in vitro studies and mouse models [34, 35]. Alter et al. recently reported that the expected cancer risk is 11-fold higher in patients with DC compared with the general population. The most frequent solid tumors were head and neck squamous cell carcinomas followed by skin and anorectal cancer [36].

Even outside DC, telomere attrition appears to cause chromosomal instability and cancer predisposition. Calado et al. [37] recently reported that patients with AA with shorter telomeres at diagnosis had a sixfold higher probability of developing clonal malignant disease following immunosuppressive therapy than patients with longer telomeres. They also showed that cultured bone marrow cells of patients with short telomeres in the presence of cytokines and high-dose granulocyte-colony stimulating factor (G-CSF) demonstrated increased telomere-free chromosomal ends and aneuploidy and translocations, including Robertsonian translocations.

Because patients with DC have been thought to be prone to myeloid malignancy, a screening for *TERT* and *TERC* mutations in patients with acute myeloid leukemia (AML) was conducted by the NIH group [38]. The authors found constitutional *TERT* mutations in 9% of patients with AML and a strong association of *TERT* mutations with the risk of cytogenetic abnormalities including trisomy 8 and inversion 16. None of the AML patients with *TERT* mutations had physical abnormalities that led to a suspicion of DC.

In addition, short telomeres have been linked to tumorigenesis of several solid tumors, including esophageal cancer, colorectal cancer, gastric cancer [39], and lung cancer [40]. Recent genome-wide studies demonstrated a higher frequency of *TERT* gene polymorphism in these patients than in normal individuals [41, 42].

6 Treatment of bone marrow failure

Bone marrow failure and immune deficiency are the most common causes of death in up to 60–70% of patients with DC. Androgen (e.g. oxymetholone) has been used to improve cytopenia in patients with DC since the 1960s. However, the mechanism of action of androgen has not been well understood until recently. Calado et al. [43] showed that in vitro exposure of normal peripheral blood cells to androgen produce higher *TERT* mRNA levels, and cells from patients who had heterozygous mutation of telomerase restored their low baseline telomerase activity to normal levels. As telomere shortening is closely associated with malignant disease, androgen therapy might prevent or postpone the development of various types of cancers. Erythropoietin and/or G-CSF combined with androgen has occasionally provided transient hematopoietic recovery to poor responders to androgen alone [44]. However, this combination should be used with caution because severe splenic peliosis and fatal rupture have been reported in two patients with DC who received simultaneous administration of androgen and G-CSF [45].

Allogeneic hematopoietic stem cell transplantation is the only curative treatment for bone marrow failure in patients with DC. However, the outcome in previous reports has been disappointing because of unacceptable transplant-related toxicities, including severe pulmonary/liver complications, especially in transplants from an alternative donor [36, 46]. To avoid these complications, non-myeloablative conditioning regimens have been recently used in several cases. Dietz et al. [47] reported encouraging results of six patients with DC who received a fludarabine-based non-myeloablative regimen. Four patients are alive, three of whom were recipients of unrelated grafts. Non-myeloablative transplants are expected to provide improvement in short-term survival. However,

longer-term follow-up is necessary because the late effects of conditioning agents and allogeneic immune responses within the recipient's organs, such as the lung and liver, remain to be clarified.

7 Future direction

Since the review article concerning DC was published by Walne et al. [14] in 2005 in this journal, many advances have occurred in the understanding of DC; however, many unsolved issues remain. Six causative genes have been identified, but mutations of these genes have been found in only half of patients with DC. Telomere-related gene mutations have been identified in patients with not only DC but also in patients with idiopathic AA, pulmonary fibrosis, and liver disease. These findings indicate that telomere-related diseases have a broad spectrum and may represent a new disease entity. A recent study demonstrated that exogenous expression of *TERC* alone can increase telomere activity and create growth potential and longevity in both *TERC* mutant and *DKC1* mutant cells [48]. More recently, Agarwal et al. [49] established induced pluripotent stem cells derived from a patient with DC and showed that the reprogrammed DC cells overcome a critical limitation in TERC levels to restore telomere maintenance and self-renewal. These findings indicate that drugs or gene therapy that can upregulate TERC activity have attractive therapeutic potential in patients with DC. Multicenter prospective studies are warranted to establish appropriate conditioning regimens aimed at reducing transplant-related mortality. We should improve not only short-term outcomes, such as hematological recovery, but also long-term effects on vital organs, especially the lungs and liver, following stem cell transplantation.

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Relapse of leukemia with loss of mismatched HLA resulting from uniparental disomy after haploidentical hematopoietic stem cell transplantation

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Brief report

Relapse of leukemia with loss of mismatched HLA resulting from uniparental disomy after haploidentical hematopoietic stem cell transplantation

Itzel Bustos Villalobos,[1] Yoshiyuki Takahashi,[1] Yoshiki Akatsuka,[2,3] Hideki Muramatsu,[1] Nobuhiro Nishio,[1] Asahito Hama,[1] Hiroshi Yagasaki,[1] Hiroh Saji,[4] Motohiro Kato,[5] Seishi Ogawa,[6] and Seiji Kojima[1]

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We investigated human leukocyte antigen (HLA) expression on leukemic cells derived from patients at diagnosis and relapse after hematopoietic stem cell transplantation (HSCT) using flow cytometry with locus-specific antibodies. Two of 3 patients who relapsed after HLA-haploidentical HSCT demonstrated loss of HLA alleles in leukemic cells at relapse; on the other hand, no loss of HLA alleles was seen in 6 patients who relapsed after HLA-identical HSCT. Single-nucleotide polymorphism array analyses of sorted leukemic cells further revealed the copy number-neutral loss of heterozygosity, namely, acquired uniparental disomy on the short arm of chromosome 6, resulting in the total loss of the mismatched HLA haplotype. These results suggest that the escape from immunosurveillance by the loss of mismatched HLA alleles may be a crucial mechanism of relapse after HLA-haploidentical HSCT. Accordingly, the status of mismatched HLA on relapsed leukemic cells should be checked before donor lymphocyte infusion. (*Blood*. 2010;115(15):3158-3161)

Introduction

Human leukocyte antigen (HLA) molecules expressed on the cell surface are required in presenting antigens to T cells. The HLA class I antigens are vital in the recognition of tumor cells by tumor-specific cytotoxic T cells. The loss of HLA class I molecules on the cell surface membrane may lead to escape from T-cell immunosurveillance and the relapse of leukemia. Previously, loss of HLA class I haplotype has been described in solid tumors.[1-3] However, there are few reports concerning HLA-haplotype loss in leukemia.[4,5]

We examined HLA class I expression in leukemic blasts from patients who relapsed after hematopoietic stem cell transplantation (HSCT) to analyze whether the loss of HLA on leukemic cells was related to the relapse after HLA-identical or haploidentical HSCT.

Methods

Patients and transplantation procedure

We identified 9 children with acute leukemia who relapsed after HSCT. Their leukemic samples were cryopreserved both at the time of the initial diagnosis and of relapse. The patients' characteristics are summarized in supplemental Table 1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Three patients received HSCT from an HLA-haploidentical family donor, and the other 6 patients received HSCT from an HLA-matched donor (4 siblings and 2 unrelated donors).

Written informed consent was given by the parents according to the protocol approved by the ethics committee of Nagoya University Graduate School of Medicine in accordance with the Declaration of Helsinki.

HLA class I expression on leukemic cells

Samples were collected at diagnosis and post-transplantation relapse. HLA expression of leukemic blasts and normal cells was analyzed by flow cytometry as previously reported.[6] Anti-HLA A2-FITC (cloneBB7.2) and anti-HLA A24-FITC (clone17a10) monoclonal antibodies were purchased from Medical & Biological Laboratories; HLA-A11 (IgM), HLA-A30, HLA-31 (IgM), HLA-25, HLA-26 (IgM), HLA-Bw6 (IgG3), and HLA-Bw4 (IgG3) antibodies were purchased from One Lambda. For leukemic cell markers, CD13-PE (IgG1) were purchased from Immunotech and CD34-APC (IgG1) were purchased from BD Biosciences. Samples were analyzed with FACSCalibur cytometer and CellQuest software. The method of genomic HLA typing was previously reported.[7]

Isolation of DNA and single nucleotide polymorphism analysis

The $CD13^+/CD34^+$ leukemic blasts were sorted by flow cytometry from bone marrow cells at the time of diagnosis and of relapse. Genomic DNA was extracted from leukemic cells sorted by a fluorescence-activated cell sorter as well as from phytohemagglutinin-stimulated patient-derived T cells and subjected to single nucleotide polymorphism (SNP) array analysis using GeneChip NspI arrays (Affymetrix) according to the manufacturer's protocol. Allele-specific copy number was detected using Copy Number Analyzer for GeneChip software as previously described.[8]

Limiting dilution-based CTLp frequency assay

The frequencies of cytotoxic T-lymphocyte precursor (CTLp) specific for the recipient-mismatched HLA molecules were analyzed using a standard limiting dilution assay.[9]

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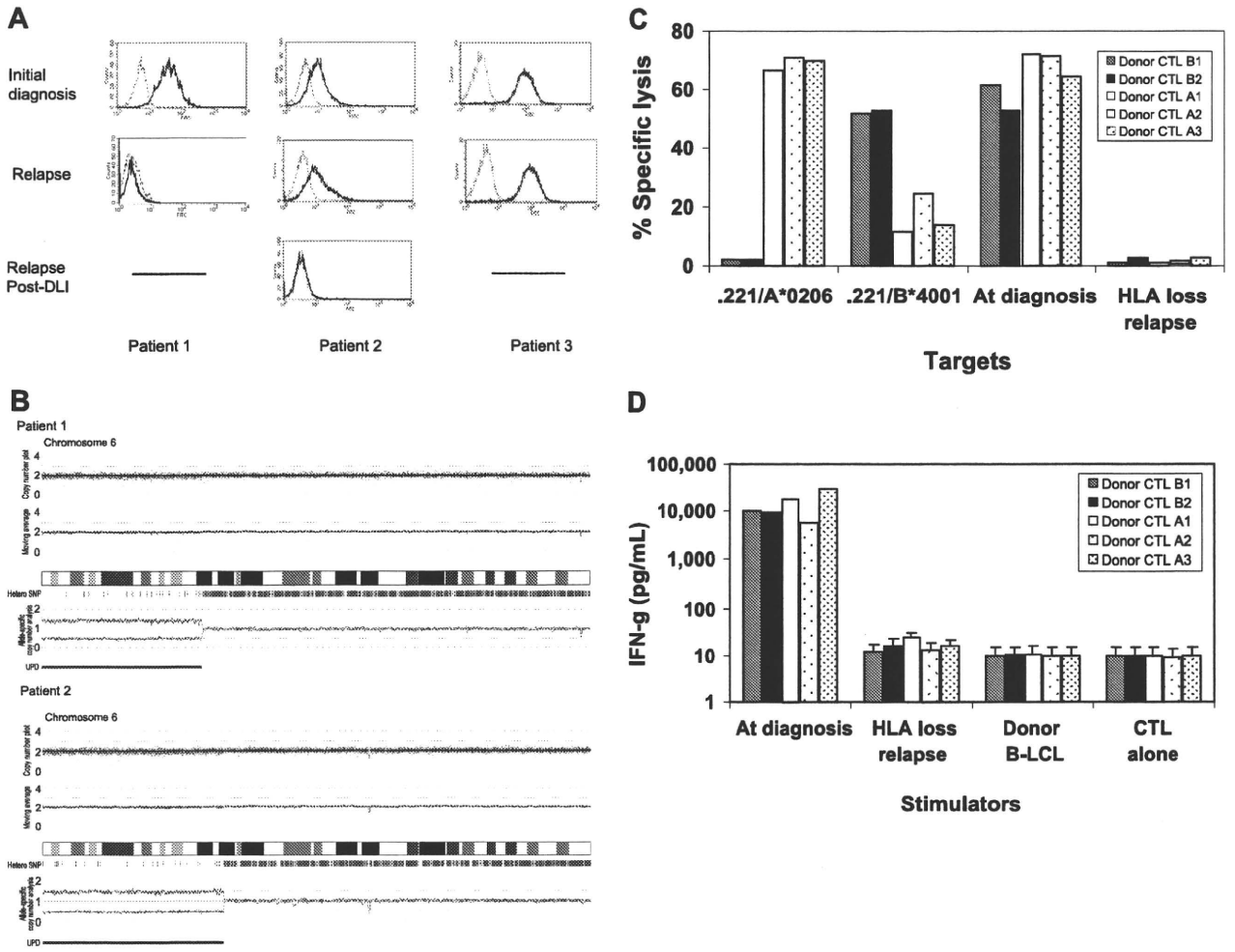

Figure 1. The loss of mismatched HLA expression on leukemic blasts caused by uniparental disomy on chromosome 6p impaired recognition and killing of donor's alloreactive cytotoxic T lymphocytes. (A) Leukemic blasts at the time of initial diagnosis and at the time of relapse after hematopoietic stem cell transplantation (HSCT) and donor lymphocyte infusion (DLI) were gated by $CD34^+$ and $CD13^+$, and then the surface expression of mismatched human leukocyte antigen (HLA) alleles was examined with anti–HLA-A2 antibodies. In 3 patients with acute myelogenous leukemia (AML) who experienced relapse after HLA-haploidentical HSCT, HLA-A2 expression was lost in patient 1 at relapse 15 months after HSCT and lost in patient 2 at second relapse 6 months after DLI. (B) Single nucleotide polymorphism (SNP) array analyses of sorted leukemic cells with the loss of an HLA allele revealed that the short arm of chromosome 6 shows copy number-neutral loss of heterozygosity or acquired uniparental disomy as detected by dissociated allele-specific copy number plots (red and blue lines at the bottom), resulting in the total loss of the mismatched HLA haplotype in both patient 1 and patient 2. The presence of acquired uniparental disomy is also indicated by normal total copy numbers with missing heterozygous SNPs (green bars) in the distal part of the short arm. (C) Recipient alloantigen-specific cytotoxic T-lymphocyte (CTL) clones were generated by a conventional cloning method from cytotoxicity-positive wells obtained in the limiting dilution assays using the donor $CD8^+$ cells as responders. Donor CTL clones A1, A2, and A3 were specific for HLA-A*0206. Donor CTL clones B1 and B3 were specific for HLA-B*4001, all of which recognize mismatched HLA alleles between the donor and recipient. Those 5 representative CTL clones were tested for HLA specificity and recognition of leukemic blasts obtained at the time of the initial diagnosis and at the time of HLA loss relapse after DLI by a standard ^{51}Cr-release assay at the effector/target ratio of 30:1. (D) Their interferon-γ production was also assessed against leukemic blasts collected at the time of diagnosis and at the time of HLA-loss relapse.

Cytotoxic assay of CTL clones against leukemic blasts and a mismatched HLA cDNA-transfected B-lymphoblastoid cell line

The remaining cells of several cytotoxicity-positive wells used for the CTLp assay for the donor were used to obtain allo-HLA–restricted CTLs. CTL clones were isolated by standard limiting dilution and expanded as previously described.[10,11]

The HLA class I–deficient 721.221 B-lymphoblastoid cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, and 1mM sodium pyruvate. Retroviral transduction was conducted as previously described.[12]

The cytotoxicity of CTL clones against target cells was analyzed by conventional chromium 51 (^{51}Cr) release assay as previously reported.[13]

CTL clones (10^4 cells/well) were mixed with the indicated stimulator cells (10^4 cells/well) in 96-well, round-bottom polypropylene plates and spun at 1200*g* for 3 minutes before overnight incubation in 200 μL of RPMI 1640 medium supplemented with 10% fetal bovine serum. On the next day, 50 μL of supernatant was collected and interferon-γ was measured by enzyme-linked immunosorbent assay with 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich).

Results and discussion

Three children with high-risk acute myelogenous leukemia (AML) received haploidentical grafts from their parents but relapsed 8, 14, and 15 months after HSCT. Patient 2 received 3 courses of donor lymphocyte infusion (DLI) for relapsed leukemia after haploidentical HSCT. After the third unmanipulated DLI (10^7 $CD3^+$/kg), she experienced acute grade-III graft-versus-host disease and achieved complete remission. However, she experienced a second relapse 6 months later. To monitor residual disease in those patients, we used flow cytometric analysis with antibodies specific for the mismatched HLA alleles between the donor and patient. Surprisingly, we found total loss of

HLA-A2 expression on $CD13^+/CD34^+$ leukemic cells from bone marrow in 2 of 3 patients who underwent HLA-haploidentical HSCT, whereas microscopic analysis showed relapse (Figure 1A). To test whether HLA class I molecules could be up-regulated, samples were cultured for 48 hours in medium supplemented with tumor necrosis factor-α or interferon-γ and measured again; however, no restoration was observed (data not shown).

Next, to examine the potential loss of genes encoding the undetectable HLA alleles, we sorted $CD13^+/CD34^+$ leukemic blasts and performed DNA genotyping. We found that, in addition to the HLA-A locus, the HLA-B, -C, and -DR loci were not encoded; only the mismatched haplotype was lost in both patients (supplemental Table 2). We then questioned whether this phenomenon would also occur in HLA-matched HSCT settings using anti-HLA class I antibodies. We did not observe any loss of HLA class I expression in any of the patients at the time of relapse (supplemental Figure 1). These results suggest that loss of HLA class I haplotype at the time of posttransplantation relapse is uncommon in HLA-matched HSCT.

To elucidate the mechanism of the loss of the mismatched HLA haplotype, we performed an SNP array analysis of genomic DNA extracted from leukemic blasts at the time of diagnosis and of relapse. Genomic DNA from patient-derived T cells was used as a reference. Leukemic cells at the time of relapse showed copy number-neutral loss of heterozygosity or an acquired uniparental disomy (UPD) of the short arm of chromosome 6 encompassing the HLA locus, whereas no allelic imbalance was identified at the time of diagnosis (Figure 1B). Loss of one allele from one parent and duplication of the remaining allele from the other parent led to UPD.[14]

In patient 2, we examined whether the number of CTLp had changed during the posttransplantation course. Limiting dilution analysis with a split-well ^{51}Cr-release assay was carried out to compare the CTLp frequencies specific for the mismatched antigens between the recipient and donor. Interestingly, the CTLp frequencies were recovered after DLI (Table 1). Restoration of CTLp after 3 DLIs could eradicate such leukemic cells, lasting for 6 months thereafter.

Next, we generated allo-HLA–restricted CTLs from $CD8^+$ cells obtained at day 520 in patient 2 and tested with the 721.221 B-lymphoblastoid cell line transfected with 1 of 3 mismatched HLA alleles (Figure 1C-D).

Despite high transplantation-related mortality resulting from severe graft-versus-host disease and posttransplantation infections, haploidentical HSCT has been widely used with the expectation of a strong graft-versus-leukemia effect.[15] However, our observation provides a possible limitation of this strategy. Indeed, 2 of 3 patients showed genomic loss of the recipient-specific HLA-haplotype, which led to escape from the graft-versus-leukemia effect and relapse of the disease.

Vago et al also reported a similar observation in 5 of 17 (29.4%) patients whose disease relapsed after haploidentical HSCT.[16] Relapsed leukemic cells may possess genomic instability that elicits genetic diversity.[17] Immunologic pressure by alloreaction to major HLA antigens may select leukemic variants of HLA class I loss, which results in the survival and proliferation of these variants.

In haploidentical HSCT, the importance of natural killer (NK)–cell alloreactivity is emphasized to achieve the graft-versus-leukemia effect.[18,19] HLA loss on leukemic blasts may in turn enhance the NK-cell alloreactivity. Our 2 patients with HLA loss had a group 1 homozygous HLA-C locus that is a suppressive killer immunoglobulin-like receptor (KIR) for NK cells and a KIR-matched donor (supplemental Table 2). Because UPD does not change the total copy number of the gene, donor NK cells should have been suppressed even after UPD occurred in these patients. Interestingly, the remaining patient who experienced relapse without HLA loss after HLA-haploidentical HSCT had a KIR-mismatched donor, so alloreactive NK cells were possibly enhanced to kill leukemic blasts with HLA loss.

Although one limitation of our study is an insufficient number of cases, our results combined with those in a recent report[16] suggest that leukemic cells occasionally escape from immunosurveillance through the loss of the mismatched HLA haplotype by the mechanism of UPD after haploidentical HSCT. DLI for relapsed AML is less effective than that for chronic myelogenous leukemia after HLA-matched HSCT.[20] However, DLI is effective even for the relapse of AML after haploidentical HSCT.[21] Evaluation of loss or down-regulation of HLA on relapsed leukemic blasts after HLA-haploidentical HSCT should be considered because DLI would probably be ineffective in patients whose leukemic cells lose HLA class I antigen.

Table 1. The CTLp frequency reactive to the recipient alloantigen in the recipient after transplantation and the donor

Samples	Maximum $CD8^+$ input*	No. of growing wells†	CTLp frequency^{-1} (95% confidence interval)
Donor	33 300	8	8.6×10^5 (1.49×10^6-5.0×10^5)
Day 100	35 500	0	UD
Day 180	17 700	0	UD
Day 300‡	86 000	0	UD
Day 520§	95 000	7	4.3×10^5 (7.2×10^5-2.5×10^5)

Purified $CD8^+$ T cells from the peripheral blood mononuclear cells obtained after transplantation from patient 2 and her donor were cultured at 2- or 3-fold serial dilutions with 33 Gy-irradiated 3×10^4 leukemic blasts cryopreserved at the time of initial diagnosis in 96-well, round-bottom plates in advanced RPMI 1640 medium supplemented with 4% pooled human serum, interleukin-6 (IL-6), and IL-7 (10 ng/mL; both from R&D Systems). The IL-2 (50 U/mL) was added on day 7 with a half medium change. For each dilution, there were at least 12 replicates. On day 14 of culture, a split-well analysis was performed for recipient-specific cytotoxicity against ^{51}Cr-radiolabeled recipient T-cell blasts, donor T-cell blasts, and leukemic blasts harvested at the time of initial diagnosis and at the time of relapse after DLI if indicated. The supernatants were measured in a γ counter after 4-hour incubation. The wells were considered to be positive for cytolytic activity if the total counts per minute released by effector cells was more than 3 SD above the control wells (mean counts per minute released by the target cells incubated with irradiated stimulator cells alone). The CTLp frequency was calculated using L-Calc software (StemCell Technologies). The CTLp frequencies reactive with recipient T-cell blasts in $CD8^+$ T cells obtained around days 100, 180, and 300 (4 months before relapse) were undetectable, whereas the CTLp frequency obtained at day 520 (1 month after the third DLI or 2 weeks after remission confirmed by bone marrow aspirate) was close to the CTLp frequency in the donor $CD8^+$ cells. Complete remission and more than 99% donor chimerism were confirmed on those days

CTLp indicates CTL precursor; and UD, undetermined because no growing wells are present.

*Number of input $CD8^+$ T cells seeded at the highest number per well.

†Number of wells out of 12 wells that received the highest $CD8^+$ cells and showed detectable growth.

‡Corresponds to 4 months before relapse.

§Corresponds to 1 month after the third DLI or 2 weeks after complete remission was confirmed by bone marrow aspirate.

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Authorship

Contribution: I.B.V. performed experiments and wrote the manuscript; Y.T. designed the research, analyzed data, and wrote the manuscript; Y.A., H.S., M.K., and S.O. performed experiments, analyzed data, and wrote the manuscript; S.K. supervised this work and wrote the manuscript; and all other authors were responsible for clinical work and critically reviewed the manuscript.

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Early Release Paper

Predicting response to immunosuppressive therapy in childhood aplastic anemia

by Nao Yoshida, Hiroshi Yagasaki, Asahito Hama, Yoshiyuki Takahashi, Yoshiyuki Kosaka, Ryoji Kobayashi, Hiromasa Yabe, Takashi Kaneko, Masahiro Tsuchida, Akira Ohara, Tatsutoshi Nakahata, and Seiji Kojima

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Predicting response to immunosuppressive therapy in childhood aplastic anemia

Running title: Predicting response in childhood aplastic anemia

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Abstract

In aplastic anemia, predictive markers of response to immunosuppressive therapy have not been well defined. We retrospectively evaluated whether clinical and laboratory findings before treatment could predict response in a pediatric cohort from the multicenter AA-97 study in Japan. Between 1997 and 2006, 312 newly diagnosed children were enrolled and treated with a combination of antithymocyte globulin and cyclosporine. In multivariate analyses, lower white blood cell count was the most significant predictive marker of better response; patients with white blood cell count $<2.0\times10^9$/L showed a higher response rate than those with white blood cell count $\geq 2.0\times10^9$/L (P=0.0003), and followed by shorter interval between diagnosis and therapy (P=0.01), and male sex (P=0.03). In conclusion, pretreatment clinical and laboratory findings influence response to therapy. The finding that response rate worsens with increasing interval between diagnosis and treatment highlights the importance of prompt immunosuppressive therapy for patients with aplastic anemia.

Key words: aplastic anemia, children, immunosuppressive therapy, predictive marker.

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Introduction

Aplastic anemia (AA) is defined as peripheral blood pancytopenia caused by bone marrow failure, and the pathogenesis is thought to involve autoimmune processes.[1-3] Several studies have confirmed immunosuppressive therapy (IST) with antithymocyte globulin (ATG) and cyclosporine (CyA) as a promising therapeutic option for patients lacking HLA-identical related donors.[4-8] Although several potential markers of IST response that appear to reflect the immune pathophysiology of AA have been suggested, mainly from adult studies,[9-11] none have been widely accepted. We have already investigated the clinical relevance of HLA, a minor population of paroxysmal nocturnal hemoglobinuria-type cells, and a specific autoantibody associated with AA in pediatric patients, finding no correlation between these markers and response to therapy.[12]

Some groups have recently shown that pretreatment laboratory variables are associated with good response to IST, but those results remain controversial, as the numbers of children included in the study was relatively small and the drugs used for IST have not been consistent.[13-15] The present study therefore evaluated whether clinical and laboratory findings before treatment could predict IST response in a large population of children with AA enrolled in a multi-center study.

Design and Methods

Patients

Between October 1997 and September 2006, a total of 312 Japanese children with AA from 118 hospitals were enrolled in the AA-97 multicenter study conducted by the Japan Childhood Aplastic Anemia Study Group. Patients with acquired AA were eligible if the following criteria were met: age <18 years; newly diagnosed disease (≤180 days) without specific prior treatment; and moderate to very severe AA. The disease was considered severe if at least 2 of the following were noted: neutrophil count $<0.5\times10^9$/L; platelet count $<20\times10^9$/L; or reticulocyte count $<20\times10^9$/L with hypocellular bone marrow.[16] AA was considered very severe if the criteria for severe disease were fulfilled and neutrophil count was $<0.2\times10^9$/L. Moderate disease was defined by at least 2 of the

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following: neutrophil count <1.0×10^9/L; platelet count <50×10^9/L; or reticulocyte count <60×10^9/L.[6] Patients with congenital AA or paroxysmal nocturnal hemoglobinuria were excluded. Allogeneic stem cell transplantation was recommended for patients with severe or very severe disease who had an HLA-matched sibling, so these patients were not included in the AA-97 study. Written informed consent was obtained from all parents and all patients >10 years old. All study protocols were approved by the ethics committee of each participating hospital. The study also conforms to the recently revised Declaration of Helsinki.

IST

All patients were treated with a combination of intravenous ATG (Lymphoglobulin; Genzyme, Cambridge, USA) at 15 mg/kg/day for 5 days and oral CyA at 6 mg/kg/day. The dose of CyA was adjusted to maintain trough levels between 100 and 200 ng/mL, and the appropriate dose was administered for at least 6 months. Granulocyte colony-stimulating factor (Filgrastim; Kirin, Tokyo, Japan) was administered intravenously or subcutaneously at 400 $\mu g/m^2$ for 3 months to only patients with very severe disease.[17] Response to IST was evaluated at 6 months after initiation of therapy. Complete response (CR) was defined as a neutrophil count >1.5 $\times10^9$/L, a platelet count >100×10^9/L, and a hemoglobin level >11.0 g/dL.[17] Partial response (PR) was defined as a neutrophil count >0.5 $\times10^9$/L, a platelet count >20×10^9/L, and a hemoglobin level >8.0 g/dL in patients with severe or very severe AA, and as a neutrophil count >1.0 $\times10^9$/L, a platelet count >30 $\times10^9$/L, and a hemoglobin level >8.0 g/dL in patients with moderate AA.[17] Overall response was defined as CR or PR at 6 months after IST.

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Statistical analyses

Parameters for univariate analyses to determine predictors of response to IST included age at diagnosis, sex, interval between diagnosis and treatment, etiology, severity of disease, white blood cell (WBC) count, neutrophil count, lymphocyte count, hemoglobin level, reticulocyte count, and platelet count. Pretreatment laboratory values were defined as the lowest value without transfusions during the 4 weeks preceding IST. Continuous variables were divided into quartile categories, and these cutoffs were used for categorical analysis. To evaluate correlations between these parameters and response, differences in continuous variables were analyzed using the Mann-Whitney *U*-test and differences in frequencies were tested using the chi-squared test or Fisher's exact test. For multivariate analyses, logistic regression modeling was performed. Important covariates in the multivariate models were chosen using stepwise variable selection procedures. Values of $P<0.05$ were considered statistically significant.

Results and Discussion

Patient characteristics are shown in Table 1. A total of 312 patients fulfilled the eligibility criteria. Median age at diagnosis was 8 years. Severity of AA was considered very severe in 156 patients, severe in 107 patients, and moderate in 49 patients. The median interval between diagnosis and treatment was 15 days. A total of 176 of the 312 (56.4%) patients improved with IST and achieved PR (n=131) or CR (n=45) at 6 months. All of them achieved transfusion independence.

To determine predictors of IST response, we compared differences in potential pretreatment variables between IST responders and non-responders. Age at diagnosis, interval between diagnosis and treatment, WBC count, neutrophil count, lymphocyte count, hemoglobin level, reticulocyte count, and platelet count were analyzed both the prevalence in categorical variables and the differences in continuous variables. In univariate analyses, WBC count, lymphocyte count, interval between diagnosis and therapy, and gender showed associations with IST response (Table 2). We

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also performed multivariate logistic regression analysis to assess the simultaneous contributions of each of the variables in predicting response. In these analyses, lower WBC count (P=0.0003), shorter interval between diagnosis and therapy (P=0.012), and male sex (P=0.036) represented significant predictors of better response (Table 2).

Boys displayed better response than girls (Figure 1A). This relationship was also observed in a retrospective European study, where a young female cohort experienced delayed recovery of bone marrow function following IST.[18] Median WBC count before treatment was significantly lower in patients who achieved response (1.9 $\times 10^9$/L) than in those who did not (2.3 $\times 10^9$/L; P= 0.007). In addition to the analysis with continuous variable, lower WBC count according to categorical analysis also associated with favorable response, with 93 of 144 patients (65%) with WBC <2.0 $\times 10^9$/L and 83 of 168 patients (49%) with WBC $\geq$2.0 $\times 10^9$/L showing improvement with IST (P=0.009; Figure 1B). When lymphocyte count was applied to the analysis instead of WBC count, a correlation between lower lymphocyte count and response to IST was also observed (Table 2); 82 of 123 patients (67%) with lymphocyte count <1.5 $\times 10^9$/L improved with IST, a significantly higher frequency than the 94 of 189 patients (50%) with lymphocyte count $\geq 1.5 \times 10^9$/L who improved with IST (P=0.004). Neither neutrophil count nor severity of disease was predictive of response.

Regarding the association between pretreatment neutrophil count and response, conflicting results have been reported. A European study reported superior response rates in children with very severe AA compared to severe AA,[5] but, unlike this observation, some studies including a recent report of a Korean cohort of adult patients have found the opposite results.[13,19] The present findings differ from those published studies, with favorable responses correlating well with lower WBC count rather than neutrophil count or disease severity. Indeed, WBC count was the strongest predictor of response to IST in multivariate analysis. In patients with AA, pretreatment WBC count may mainly reflect the size of lymphocyte populations, due to the severe neutropenia in this condition. These results suggest that poor response to IST might possibly be ascribed to higher WBC count, that is, a relative increase in lymphocytes. Given the dramatic effects of T-cell suppressants including ATG

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and CyA on in vivo hematopoiesis, autoreactive T-cell responses against hematopoietic stem cells have been suggested to play a major role in the pathogenesis of AA, and in vitro studies have also supplied supportive evidence for this idea. Early experiments demonstrated inhibitory effects of autologous lymphocytes on hematopoietic progenitor cell growth through overproduction of cytokines such as interferon-γ and tumor necrosis factor-α by activated cytotoxic T cells in AA patients.[20-22] More recently, oligoclonal T-cell expansions have been described in AA patients, disappearing with clinical improvement following IST.[23] Taking our results and previous findings together, a higher WBC count before treatment may indicate the presence of numerous autoreactive T cells that need to be eliminated and thus a high potential to destroy marrow function through lymphocytes, rather than better residual marrow function. In this scenario, patients with a lower WBC count could be seen to have a better probability of hematopoietic recovery following IST.

We identified a significantly inverse correlation between response and interval between diagnosis and treatment; median intervals among responders and non-responders were 13 and 19 days, respectively (P=0.002). In categorical analysis, response rates of patients with intervals <30 and ≥30 days were 60% and 43%, respectively (P=0.013). Figure 1C clearly indicates the inverse relationship. Notably, response rates to IST were considerably low among AA patients with long-standing disease; only 35% of patients treated ≥90 days after diagnosis responded, suggesting that patients with this condition may receive irreversible damage to hematopoietic progenitor cells or stromal elements that progresses over time, possibly due to immune attack through autoreactivated lymphocytes. The present study indicates the importance of prompt IST therapy for patients with AA. We therefore recommend offering IST as soon as possible in all children with AA who lack a matched sibling donor.

Other variables did not differ significantly between responders and non-responders (Table 2). Particularly with regard to reticulocyte count, 122 patients showed reticulocyte count >25 $\times 10^9$/L, of whom 67 (55%) responded to IST, and 186 patients had reticulocyte count ≤25$\times 10^9$/L, of whom 107 (58%) responded to IST. Correlations of higher reticulocyte count and higher lymphocyte count at

initial diagnosis with better response to IST in patients of all ages have recently been described by the National Institutes of Health (NIH) group.[15] However, when the same analysis was applied to their 77 pediatric patients, lymphocyte count was not predictive.[14] More recently, another relatively small study in adults with AA found no such association.[13] These studies were limited by inconsistency of regimens used for IST. The current study investigated a large cohort of children with AA treated using a unified regimen, but failed to confirm any correlation between reticulocyte count and response to IST, suggesting a limited contribution of this clinical parameter to the prediction of hematopoietic recovery, at least in children.

In conclusion, pretreatment clinical and laboratory findings influence response to IST. Favorable response correlates better with lower WBC count than with neutrophil count or disease severity, and this blood count parameter might help in clinically assessing bone marrow function. Unlike the situation in adult AA, reticulocyte count is not predictive of response to IST in pediatric patients. IST should be started as soon as possible after diagnosis of AA, given that the response rate worsens with increasing interval between diagnosis and treatment.

Authorship and Disclosures

NY and SK designed and performed the research; NY, HY, YT, and SK analyzed data; YK, RK, HY, TK, MT, AO, TN, and SK collected and managed clinical data; and NY and SK wrote the paper.

The authors reported no potential conflicts of interest.

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